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METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS*

DESCRIPTION

Field of the Invention

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

Background of the Invention

M. tuberculosis can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing it's uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high-level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for *M. tuberculosis*, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular biological methods for the detection of M. tuberculosis and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR–based or probe-based. These tests are used

primarily on AFB smear-positive samples. Since the presence of *M. tuberculosis* has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. Tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533[a]
2.	Isoniazid	katG gene	codon 275/315/328[b]
3.	Isoniazid	mabA gene	unknown[a]
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A[e]
6.	Pyrazinamide	pncA gene	codon 47/85[f]
7.	Ethambutol	embB gene	codon 306[g]
8.	Streptomycin	rpsL/s12 gene	codon 43/88[h]
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95[j]

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in it's present form.

Abstracts of the Interscience Conference on Antimicrobial Agents and Chemotherapy", vol. 34, page 163 (1994) describes the application of automated DNA sequence analysis of *hsp65* to speciation of isolates previously-identified as being *M. tuberculosis*.

Thus, there remains a need for a method for detecting antibiotic-resistance mutations in clinical *M. tuberculosis* sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in *M. tuberculosis*.

Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes. Using these primer sets and the OPENGENE™ automated DNA sequencing system, a protocol has been developed which permits both the rapid detection of *M. tuberculosis* and the identification of antibiotic resistance–associated mutations in a series of gene targets. The present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic-resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture *M. tuberculosis* and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

Brief Description to the Figures

Fig. 1 shows known testing protocols for *M. tuberculosis*; and

Fig. 2 shows a hierarchical assay scheme for evaluating *M. tuberculosis* type in accordance with the invention.

Detailed Description of the Invention

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are know, some of which require prior amplification and some of which do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

Primers

rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ. ID NO. 1

rpoB-R amplification primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 2

rpoB-5s sequencing primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ ID NO. 3

rpoB-3s sequencing primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 4

SEQ. ID. NO. 5

2161 aaaccgacga catcgaccac ttcggcaacc gccgcctgcg tacggtcggc gagctgatcc
2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca
2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg
2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc
2401 tgtcggggtt gacccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg
2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga
2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcggtg tacgcgcggg
2581 tcaacccgtt cgggttcatc gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg

katG (isoniazid resistance)

katG-F amplification primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 6

katG-R amplification primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 7

katG-5s sequencing primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 8

katG-3s sequencing primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 9

SEQ ID NO. 10

661 gctcggcgat gagcgttaca gcggtaagcg ggatctggag aacccgctgg ccgcggtgca
721 gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc
781 ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc
841 gctgatcgtc ggcggtcaca ctttcggtaa g**acc**catggc gccggcccgg ccgatctggt
901 cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta
961 tggcaccgga accggtaagg acgcgatcac c**agc**ggcatc gaggtcgtat ggacgaacac
1021 cccgacgaaa **tgg**gacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac

1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat
1141 cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct
1201 gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt
1261 ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc

oxyR-aphC intergenic region (PR)

PR-F amplification primer, 20-mer, bp451-470
5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 11
PR-R amplification primer, 20-mer, bp687-668
5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 12
PR-5s sequencing primer, 20-mer, bp451-470
5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 13
PR-3s sequencing primer, 20-mer, bp687-668
5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 14

SEQ ID NO. 15
361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc
421 tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg
481 cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt
541 gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc
601 attgtccgct ttgatgatga ggagagtcat gccactgcta accattggcg atcaattccc
661 cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc
721 cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt

mabA (isoniazid resistance)

mabA-F amplification primer, 20-mer, bp56-75
5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 16
mabA-R amplification primer, 20-mer, bp303-284
5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 17
mabA-5s sequencing primer, 20-mer, bp56-75
5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 18

mabA-3s sequencing primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 19

SEQ ID NO. 20

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1   agcgcgacat acctgctgcg caattcgtag ggcgtcaata caccccgcagc cagggcctcg
61  ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg
121 agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca
181 taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac
241 tgaaggggcc aaaccccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg
301 gatcgggctg gcgatcgcac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca
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rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 21

s12-R amplification primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO 22

s12-5s sequencing primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 23

s12-3s sequencing primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO. 24

SEQ ID NO. 25

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1   cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag
61  gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac
121 accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcgt gaagttgacg
181 agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg
241 atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc
301 cgcggttcgc tggatacgca gggtgtcaag aaccgcaaac aggcacgcag ccgttacggc
361 gctaagaagg agaagggctg atgccacgca aggggcccgc gcccaagcgt ccgttggtca
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16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 26

16S-R amplification primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 27

16S-5s sequencing primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 28

16S-3s sequencing primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 29

SEQ ID NO. 30

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1   cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta ataccggata
61  ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg
121 gcctatcagc ttgttggtgg ggtgacg
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embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 31

embB-R amplification primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 32

embB-5s sequencing primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 33

embB-3s sequencing primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 34

SEQ ID NO. 35

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7741 cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt
7801 gatattcggc ttcctgctct ggcatgtcat cggcgcgaat tcgtcggacg acggctacat
7861 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt
7921 cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt
7981 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct
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pncA (pyrazinamide resistance)

pncA-F amplification primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 36

pncA-F amplification primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 37

pncA-5s sequencing primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 38

pncA-3s sequencing primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 39

SEQ ID. NO. 40

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1   atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctcgctggcg
61  gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac
121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc
181 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac
241 ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc
301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg
361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc
421 cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca
481 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc
541 gagttggttt gcagctcctg a
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gyrA (fluoroquinilone/ciprofloxacin resistance)

gyrA-F amplification primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 41

gyrA-R amplification primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 42

gyrA-5s sequencing primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 43

gyrA-3s sequencing primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 44

SEQ ID NO. 45

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2341 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc
2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca
2461 tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa
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2521 gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat
2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg
2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc
2701 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt

23S (macrolide/azithromycin resistance)

23S-F amplification primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 46

23S-R amplification primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 47

23S-5s sequencing primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 48

23S-3s sequencing primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 49

SEQ ID NO. 50

2401 gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg
2461 taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg
2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga
2581 ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt
2641 tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OPENGENE™ sequencer), at least one of the sequencing primers is preferably labeled with a flourescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as CY5.0 OR CY5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers), and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix		**1 PCR**	**10 PCRs**	**final conc. / PCR**
genomic DNA (~0.5fM)	(20ng/ul)	1.0ul		20ng
10X PCR buffer I		2.5ul	25.0ul	1X
2.5mM dNTP mix	(1:1:1:1)	2.5ul	25.0ul	250uM
DMSO		1.3ul	13.0ul	5%
Taq DNA polymerase (1U)		0.2ul	2.0ul	1 unit
molecular grade water		16.5ul		165.0ul
MTB gene primers	(10uM)	1.0ul	10.0ul	10pmol per primer
total volume per PCR		25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using

the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

<u>Cycle sequencing mastermix</u>

rpoB template	2.0ul
10X VGI Sequenace ™ buffer	2.5ul

DMSO	3.5ul
2.5uM dye-sequencing primer	2.0ul
PCR grade water	9.0ul
1:10 diluted Thermosequenase	0.5 ul
total volume	22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the CLIPPER™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

The CLIPPER™ sequencer is set-up as described in the *OPENGENE Automated DNA Sequencing System User Manual.* Run parameters for the CLIPPER™ sequencer are 54°C/ 1300volts/ 0.5sec sampling/35min run/50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with CY5.0 and CY5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GENELIBRARIAN™. This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GENELIBRARIAN™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis.* In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A limitation of the AFB stain methodology is it's limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect M. tuberculosis (rpoB), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat M. tuberculosis infections (rpoB, katG, rpsL/s12, PR, embB, pncA)

and detect other species of mycobacteria (23S) in the absence of *M. tuberculosis* (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (rpoB), isoniazid (katG), streptomycin (rpsL/s12) and azithromycin (23S). In addition the rpoB amplification indicates the presence of *M. tuberculosis* and in the absence of rpoB amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (embB), pyrazinamide (pncA) and ciprofloxacin (gyrA). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of *M. tuberculosis* from both AFB smear-positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada. Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the rpsL/s12 gene in four isolates. Parallel antibiotic resistance-associated mutations in the rpoB (rifampin), katG (isoniazid), PR (isoniazid),

embB (ethambutol), pncA (pyrazinamide) and gyrA (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference for all states which allow such incorporation.

[a] **DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. Antimicrob Agents Chemother 38: 2380-2386.**

[b] **WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of Mycobacterium tuberculosis complex from Africa. Antimicrob Agents Chemother 41: 1601-1603.**

[c] **S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and –susceptible Mycobacterium tuberculosis complex organisms recovered from diseased humans and animals in diverse localities. Antimicrob Agents Chemother 41: 600-606.**

[d] **A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at the reference laboratory level. Antimicrob Agents Chemother 35: 719-723.**

[e] **C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.**

[f] **C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.**

[g] **MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in Mycobacterium smegmatis. Antimicrob Agents Chemother 41: 2629-2633.**

[h] **A Scorpio et al. (1997). Characaterisation of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 41: 540-543.**

i C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis. J Infect Disease 174: 1127-1130.

j KA Nash et al. (1995). Genetic basis of macrolide resistance in Mycobacterium avium isolated from patients with disseminated disease. Antimicrob Agents Chemother 39: 2625-2630.

Table 1

gene (antibiotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
rpoB (rifampin)	cac526tac, His526Tyr	tcg553ttg, Ser553Leu	cac526gac, His526Asp	tcg553ttg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpsL/s12 (streptomycin)	wt	aag43agg, Lys43Arg	aag43agg, Lys43Arg	aag88agg, Lys88Arg	aag43agg, Lys43Arg
16s/rrs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	gtc292ttc, val292phe	wt	wt	wt
pncA (pyrazinamide)	tcc65tct, Ser65Ser	wt	att133aat, Ile133Asn	wt	tcc65tct, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt